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Crystal-induced inflammation in the rat subcutaneous air-pouch

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1 Monosodium urate (MSU) and calcium pyrophosphate dihydrate (CPPD) crystals initiated acute inflammatory reactions characterized by increased plasma extravasation and polymorphonuclear leukocyte (PMNL) accumulation in the rat subcutaneous air-pouch.

2 Pretreatment of rats with colchicine (1 mg kg^{-1}, s.c.) inhibited PMNL accumulation induced by either crystal type but had a greater inhibitory effect on MSU-induced plasma extravasation compared with that induced by CPPD crystals.

3 Colchicine (1 mg kg^{-1}, s.c.) did not reduce histamine-induced plasma extravasation in the air-pouch.

4 The lipoxygenase product of arachidonic acid metabolism, leukotriene B_4 (LTB_4), was detected in MSU-induced exudates but not in CPPD-induced exudates.

5 Pretreatment of rats with colchicine (1 mg kg^{-1}, s.c.) inhibited LTB_4 production in MSU-induced exudates.

Introduction

The synovial deposition of monosodium urate (MSU) and calcium pyrophosphate dihydrate (CPPD) crystals is associated with gout (McCarty & Hollander, 1961) and pseudogout (Kohn *et al.*, 1962), respectively. Both crystal types initiate acute inflammatory responses in models of inflammation (McCarty *et al.*, 1966: Denko & Whitehouse, 1976; Glatt *et al.*, 1979) which are characterized by increases in vascular permeability and polymorphonuclear leukocyte (PMNL) accumulation. Colchicine demonstrates anti-inflammatory activity both in models of crystal-induced inflammation (Denko & Whitehouse, 1976; Glatt *et al.*, 1979) and against non-crystalline inflammatory stimuli such as *Staphylococcus aureus* (Malawista & Andriole, 1976) and carrageenan (Vinegar *et al.*, 1981; Simmons *et al.*, 1983). However, clinically colchicine is almost exclusively effective in the treatment of gout rather than other inflammatory joint diseases (Wallace *et al.*, 1967). The reason for this selective action in gout is unknown.

The lipoxygenase metabolite of arachidonic acid leukotriene B_4 (Borgeat *et al.*, 1976) has been detected in synovial fluid aspirated from the knees of patients with gout (Rae *et al.*, 1982) and in PMNL supernatants following their incubation with MSU crystals (Serhan *et al.*, 1984). LTB_4 increases vascular permeability (Wedmore & Williams, 1981; Bray *et al.*, 1981a) and is chemotactic both *in vivo* (Smith *et al.*, 1980; Bray *et al.*, 1981b; Bhattacherjee *et al.*, 1981) and *in vitro* (Goetzl & Pickett, 1980; Palmer *et al.*, 1980) for PMNLs.

This current study was designed to test whether MSU and CPPD crystals produce inflammatory changes in the rat air-pouch model of inflammation; to see if leukotriene B_4 is generated in response to these crystals and whether colchicine exhibits a selective anti-inflammatory action in this model.

Methods

Animals

Male Wistar rats (150–180 g) were purchased from the New South Wales Institute of Technology Research Laboratory. Animals were maintained on standard laboratory diet and water *ad libitum*.

Rat air-pouch

Air-pouches were formed based on methods described

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by Edwards *et al.* (1981). Rats were anaesthetized with methihexitone sodium (25 mg kg^{-1}, i.p.) and were injected subcutaneously with 15 ml of sterile air on their dorsal surface just behind the scapulae. This procedure was repeated 2 and 4 days after the first injection. Rats were used in experiments between 7 and 10 days following the first injection.

Crystal-induced inflammation

Rats with air-pouches were injected subcutaneously with either colchicine (1 mg kg^{-1}) or saline (0.5 ml). Thirty minutes later the animals were anaesthetized with methihexitone sodium (25 mg kg^{-1}, i.p.) and then injected intravenously with Evans blue dye (2.5% wt/vol; 2.0 ml kg^{-1}) which binds to circulating plasma albumin and acts as a marker for plasma extravasation. An 18 gauge polythene cannula was inserted into the air-pouch via a concentric steel needle which was removed to leave the cannula in place. Fifteen ml of a suspension of either MSU or CPPD crystals (10 mg ml^{-1}) in normal saline or saline alone was injected into the air-pouch via the cannula. A 1 ml sample of air-pouch fluid was removed immediately and collected into EDTA (10 mM final concentration) and 50 µl of this sample was placed into a separate tube for a cell count and the remainder centrifuged (7,800 *g* for 2 min). The exudate was frozen at − 20°C before assaying for LTB_4 and the concentration of Evans blue dye. Further samples were collected and treated in an identical manner at 1, 2, 4, 6 and 8 h.

Histamine-induced plasma extravasation

Animals were prepared as described for 'crystal-induced inflammation'. Following the injection of Evans blue dye the animals were injected with 10 ml of histamine (100 µg ml^{-1}) via a 23 g needle into the air pouch. Fourty five minutes later the animals were killed with an overdose of pentobarbitone, the air-pouch was opened with scissors and the exudate fluid in the pouch removed with a plastic pipette into a tube containing EDTA (10 mM final concentration).

Measurement of plasma extravasation

Plasma extravasation was determined by the optical absorbance at 620 nm of all exudate samples. The rate of increase of absorbance per hour was calculated and plotted against the time of recovery of the exudate from the air-pouch.

Polymorphonuclear leukocyte accumulation

The number of PMNLs in the exudate was measured by light microscopy following the staining of exudates with gentian violet (0.01% crystal violet, 1% acetic acid, 99% water). The rate of increase in the number of PMNLs per ml of exudate was calculated and plotted against the time of recovery of the exudate from the air-pouch.

Leukotriene B_4 extraction and assay

Five hundred µl of exudate was applied to a C-18 Sep-pak (Millipore-Waters Pty Ltd) under gravity and the column washed with 20 ml of water followed by 2 ml of hexane and 2 ml of methylene chloride. The column was eluted with 2 ml of methanol which was dried under nitrogen then reconstituted in 500 µl of the h.p.l.c. mobile phase (63% methanol, 37% water, 0.02% acetic acid adjusted to pH 6.8 with 30% ammonium hydroxide).

The h.p.l.c. system consisted of a Waters model 510 pump, U6k injector and model 481 detector with a 25 cm Altex Ultrasil ODS column. Identification of LTB_4 was by comparison of elution time with that of synthetically prepared LTB_4. Quantitation was performed by measuring optical absorbance at 270 nm followed by interpolation on a standard curve prepared using synthetic LTB_4.

Drugs and chemicals

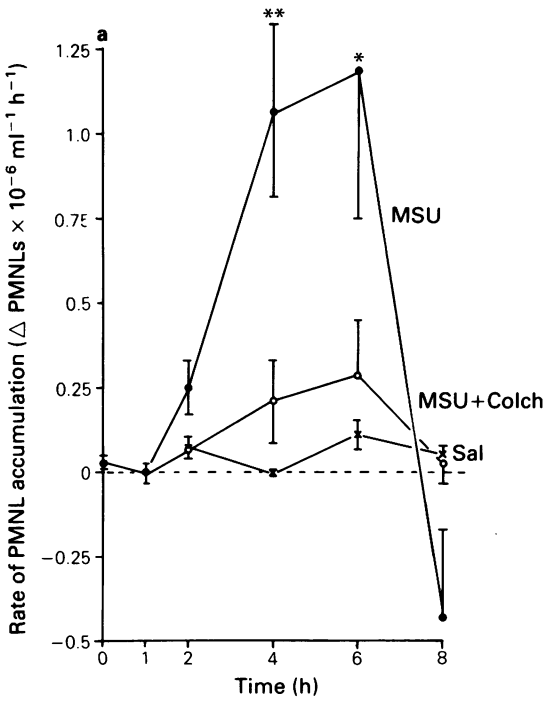
MSU and CPPD crystals were prepared according to the methods of Denko & Whitehouse (1976). Methihexitone sodium (Brietal) was purchased from Eli Lilly (Australia) Pty Ltd, Sydney and Evans blue dye from Sigma Chemical Co. St. Louis, Mo. USA. Colchicine was a gift from Fisons (Australia) Pty Ltd, Sydney and leukotriene B_4 from Dr J. Rokach, Merck, Frosst Canada Inc.

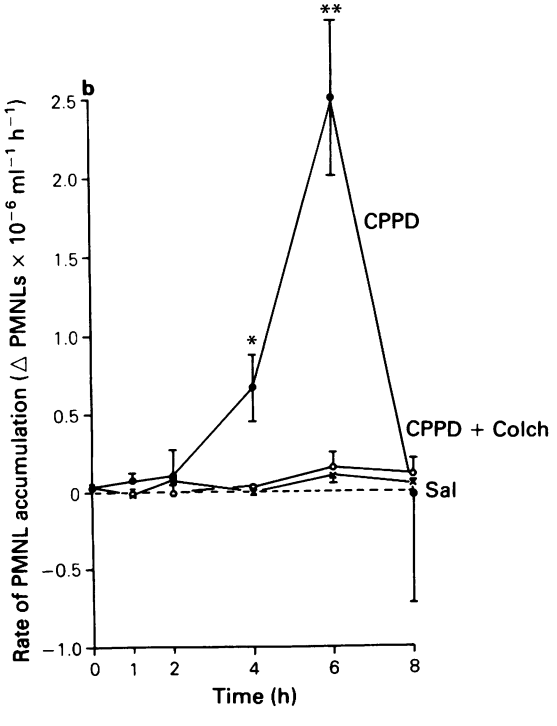
Statistics

Results are presented as mean ± s.e.mean and compared for statistical significance by Student's *t* test.

Results

The injection of a suspension of either MSU (Figure 1a) or CPPD (Figure 1b) crystals into the rat air-pouch induced significant PMNL accumulation compared with saline injected animals. Although the maximum rate of PMNL accumulation occurred at 6 h with both crystal types there were a greater number of PMNLs in CPPD-induced exudates compared with MSU-induced exudates. No further PMNL accumulation was evident at 8 h. However, both MSU and CPPD-induced exudates still contained significant numbers of PMNLs ($3.7 \pm 0.6 \times 10^6$ PMNLs ml^{-1} and $6.5 \pm 0.8 \times 10^6$ PMNLs ml^{-1}, respectively). Col-





chicine significantly reduced PMNL accumulation induced by either crystal type to the levels found in saline injected animals.

Both MSU (Figure 2a) and CPPD (Figure 2b) crystals induced significant plasma extravasation into the rat air-pouch compared with saline injected controls which reached a maximum rate at 6 h. Unlike PMNL accumulation the rate of plasma extravasation was maintained at 8 h. Colchicine reduced MSU-induced plasma extravasation to below control values at all time points whereas against CPPD-induced plasma extravasation the inhibition was never complete and was significant only at 4 and 6 h. Colchicine did not reduce plasma extravasation induced by histamine ($100\,\mu g\,ml^{-1}$) in the air-pouch over a 45 min period (Figure 3).

Both MSU and CPPD-induced exudates collected at 4 h were examined for the presence of LTB_4 (Figure 4). The MSU but not the CPPD-induced exudate contained a significant amount of LTB_4 compared with saline injected animals. The increase in LTB_4 production was reduced to control levels in colchicine pretreated animals. The time course for LTB_4 generation induced by MSU is shown in Figure 5. The presence of LTB_4 did not reach a significant level until 4 h after injection of MSU crystals. In contrast significant numbers of PMNLs had accumulated by 2 h.

Discussion

The injection of suspensions of MSU or CPPD crystals into the rat subcutaneous air-pouch resulted in the initiation of acute inflammatory reactions. Both crystal types produced similar inflammatory reactions in terms of the magnitude and time courses of PMNL accumulation and plasma extravasation. We observed only a marginally greater reduction in MSU-induced plasma extravasation by colchicine compared with CPPD-induced plasma extravasation and no dif-

Figure 1 The rate of polymorphonuclear leukocyte (PMNL) accumulation into the rat subcutaneous air-pouch induced by (a) monosodium urate (MSU) crystals ($10\,mg\,ml^{-1}$, ●) and (b) calcium pyrophosphate dihydrate (CPPD) crystals ($10\,mg\,ml^{-1}$, ●) and the effects of pretreatment of animals with colchicine (Colch, $1\,mg\,kg^{-1}$ s.c., ○). One ml samples of exudate fluid were withdrawn from the air-pouch at defined time points after the injection of 15 ml of a suspension of MSU or CPPD crystals in saline or 15 ml of saline alone (Sal, ×) into the air-pouch. The number and type of cells present was determined by light microscopy after staining the cells with gentian violet. The symbols shown are mean values and the vertical lines represent s.e.mean, $n = 6$ animals at each point. $^*P<0.05$; $^{**}P<0.01$.

ference in the ability of colchicine to reduce PMNL accumulation induced by either crystal type. However, a major difference in the inflammatory response produced by the two crystal types was the presence of LTB_4 in MSU-induced exudates but not in CPPD-induced exudates. This observation together with the clinical finding of raised LTB_4 levels in the synovial fluid of patients with gout (Rae *et al.*, 1982) and the demonstration of LTB_4 generation by PMNLs *in vitro* when incubated with MSU crystals (Serhan *et al.*, 1984) provides a clear association between MSU crystals, PMNLs and the production of LTB_4. Whether or not the differential clinical efficacy of colchicine in gout is related to this association remains to be determined.

Increased vascular permeability resulting in plasma protein extravasation can result from the direct action of a mediator on venular endothelial cells or indirectly via an interaction between PMNLs and venular endothelial cells (Wedmore & Williams, 1981). We have shown that colchicine does not reduce plasma extravasation induced by histamine but totally inhibits MSU-induced plasma extravasation and partially reduces that induced by CPPD crystals. These results indicate that colchicine does not have a non-specific effect on vascular endothelial cells to prevent an increase in vascular permeability but that it interferes with a PMNL-endothelial cell interaction to prevent both PMNL accumulation and plasma extravasation. Furthermore, it would appear that MSU-induced plasma extravasation occurred solely via a PMNL-dependent mechanism whereas plasma extravasation induced by CPPD crystals exhibited a component which was independent of PMNLs.

The presence of LTB_4 in MSU-induced inflammatory exudates, which is a PMNL-dependent permeability-increasing mediator (Wedmore & Williams, 1981), suggests that LTB_4 is mediating the increase in plasma extravasation induced by MSU crystals. However, two pieces of evidence argue against this proposal. Firstly, the maximum concentration of LTB_4 detected in the inflammatory exudate was 0.012 μM which by comparison with the work of other groups would barely increase plasma extravasation above baseline values. For example Wedmore & Williams (1981) used a dose range of approximately

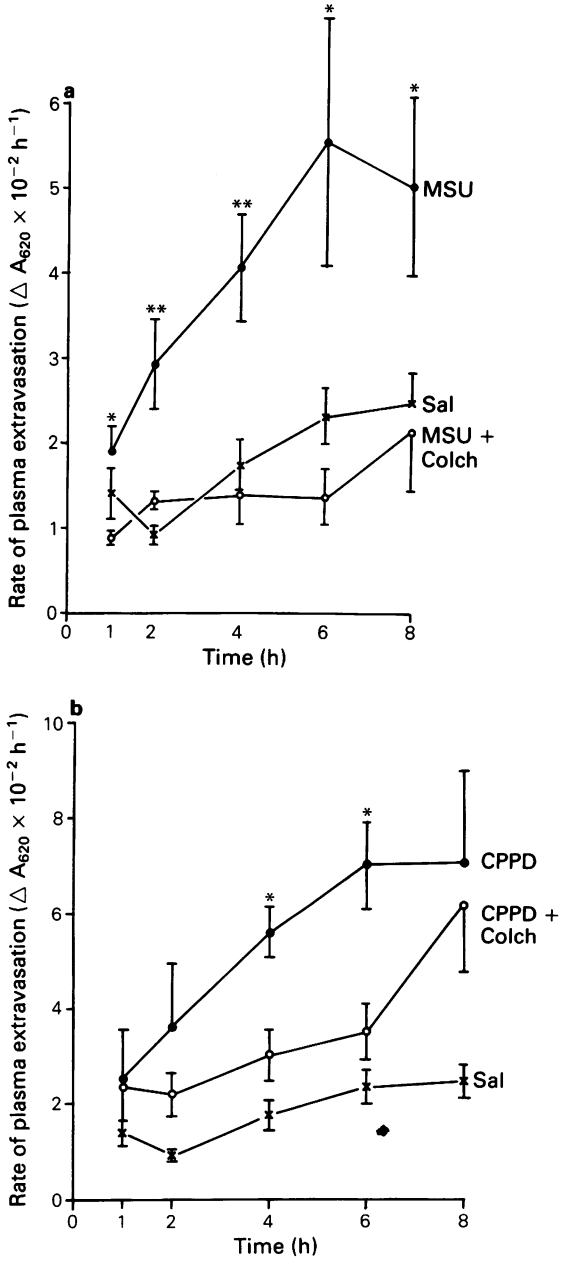


Figure 2 The rate of plasma protein extravasation into the rat subcutaneous air-pouch induced by (a) monosodium urate (MSU) crystals (10 mg ml^{-1}, ●) and (b) calcium pyrophosphate dihydrate (CPPD) crystals (10 mg ml^{-1}, ●) and the effects of pretreatment of animals with colchicine (Colch, 1 mg kg^{-1} s.c., ○). Rats were injected intravenously with Evans blue dye to act as a marker for plasma protein extravasation into the air-pouch. One minute later they were injected with 15 ml of a suspension of MSU or CPPD crystals in saline or 15 ml of saline alone (Sal, ×) into the air-pouch. One ml samples of exudate fluid were withdrawn from the air-pouch at defined time intervals and the concentration of blue dye in the exudate determined by the optical absorbance of the sample of 620 nm. The symbols shown are mean values and the vertical lines represent s.e.mean, $n = 6$ animals at each time point. $*P < 0.05$; $**P < 0.01$.

0.02–2.0 μM; Bray *et al.* (1981) used from 0.03–3.0 μM and Bjork *et al.* (1982) used from 0.15–5 μM. Secondly, LTB_4 was not detectable in the exudate fluid until 4 h following the injection of MSU crystals whereas significant increases in both plasma extravasation and PMNL accumulation were evident at 2 h. This does not totally exclude a role for LTB_4 in mediating PMNL accumulation and plasma extravasation in this model as LTB_4 may be acting to propogate these responses initiated by other mediators generated earlier in the response to MSU crystals.

The mechanism by which colchicine inhibited LTB_4 generation induced by MSU crystals in this model is not clear from these experiments. It has previously been reported that colchicine may have a direct effect on the metabolism of arachidonic acid via interference with the lipoxygenase enzyme system (Serhan *et al.*, 1984). However, results from a later study by Reibman *et al.* (1986), who used the calcium ionophore A23187 as a stimulus for LTB_4 generation by PMNLs, suggested that inhibition of LTB_4 production is related to the disruption of microtubules by colchicine. If we assume that PMNLs accumulating in the air-pouch are the source of LTB_4 in MSU-induced exudates, then the inhibition of PMNL accumulation by colchicine would account for the inhibition of LTB_4 production seen in this model.

Rae *et al.* (1982) demonstrated that MSU crystals

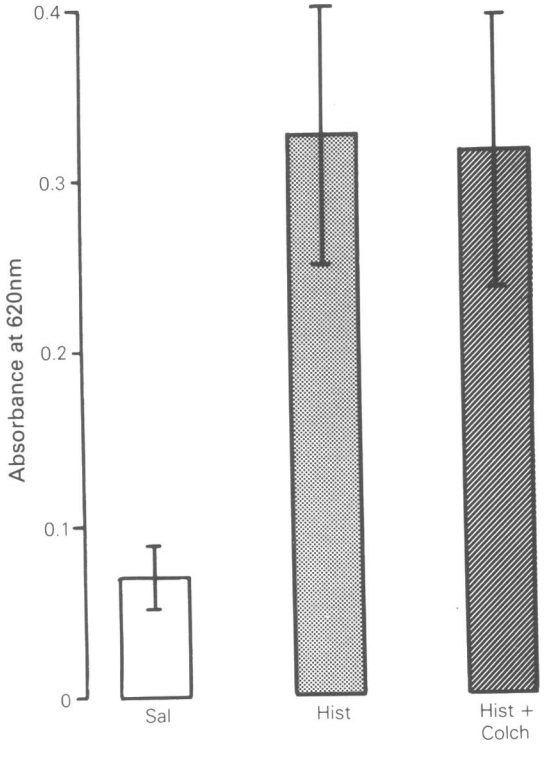


Figure 3 Pretreatment of rats with colchicine (Colch, 1 mg kg^{-1} s.c.) did not reduce plasma extravasation induced by histamine (Hist, 100 μg ml^{-1}). Rats were injected intravenously with Evans blue dye to act as a marker for plasma protein extravasation into the air-pouch. One minute later they were injected with 10 ml of histamine or saline (Sal) into the air-pouch; 45 min later the animal was killed and the concentration of blue dye in the exudate determined by the optical absorbance of the sample at 620 nm. Each column represents the mean value and the vertical lines show s.e.mean, $n = 6$ animals.

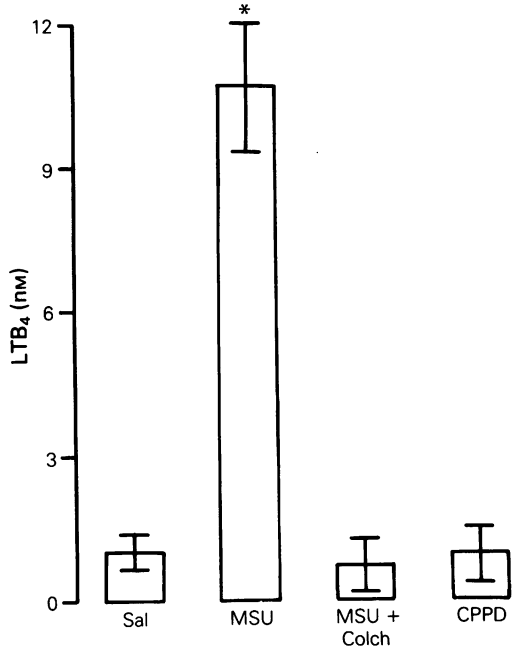


Figure 4 The presence of leukotriene B_4 (LTB_4) in air-pouch exudate fluid recovered 4 h after an injection of 15 ml of saline (Sal), monosodium urate crystals (MSU, 10 mg ml^{-1}), MSU crystals (10 mg ml^{-1}) following pretreatment of rats with colchicine (Colch, 1 mg kg^{-1}) and calcium pyrophosphate dihydrate crystals (CPPD, 10 mg ml^{-1}) was assessed by high performance liquid chromatography. Aliquots, 500 μl, of exudate fluid were extracted on C-18 Sep-pak cartridges before injection onto a 25 cm Altex 5 μm C-18 column. The identification of material was by an identical rentention time with a known standard. Quantitation was by interpolation on a standard curve constructed using known quantities of LTB_4. Each column represents mean values and vertical lines show s.e.mean. $n = 6$ animals in each group. $^*P < 0.05$.

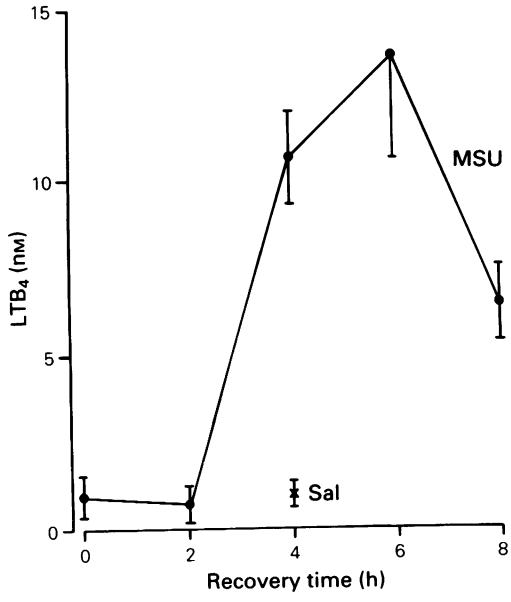


Figure 5 Time course for the generation of leukotriene B_4 (LTB_4) in monosodium urate (MSU) crystal-induced inflammatory exudates in the rat air-pouch. The amount of LTB_4 in saline (Sal)-induced exudate at 4 h is shown for comparison. The symbols shown are mean values and the vertical lines represent s.e.mean. $n = 6$ animals at each time point.

were not only a stimulus for LTB_4 production by PMNLs but that the crystals also prevented the metabolism of LTB_4 to its more polar derivatives. It is conceivable that both CPPD and MSU crystals stimulate LTB_4 production by PMNLs, but LTB_4 is only detected in MSU-induced exudates because its further metabolism has been blocked by MSU crystals. In addition, one may then suggest that the inhibitory effect of colchicine on LTB_4 production seen here (Figure 4) could be attributed to colchicine overcoming the inhibitory effect of MSU on the further metabolism of LTB_4.

It is recognised that physico-chemical properties of crystals affect their potential to initiate an inflammatory reaction (Dieppe & Calvert, 1983). We have now demonstrated that the chemical nature of the crystal can also affect the profile of mediators generated in response to the presence of the crystal. The association between LTB_4 and gout clinically (Rae *et al.*, 1982) and the demonstration here of an association between LTB_4 and MSU crystal-induced inflammation suggests that compounds with anti-lipoxygenase activity may be particularly effective in the treatment of gout.

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